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USAARL REPORT NO. 69-15

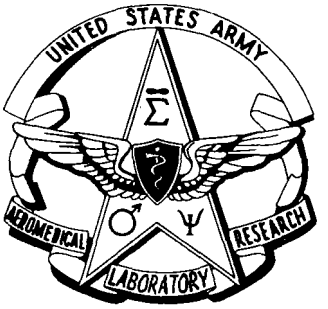
FORMS OF CLOSED CIRCULAR DNA IN RAT LIVER DURING REGENERATION AND FOLLOWING AMINOAZO-DYE CARCINOGENESIS

By

James G. Wetmur, CPT, MSC
Charles R. Wilson, SP5

JUNE 1969

U. S. ARMY AEROMEDICAL RESEARCH LABORATORY
Fort Rucker, Alabama



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ACKNOWLEDGEMENTS

We should like to thank Dr. Beischer of the Naval Aerospace Medical Institute for permitting our free access to a Phillips EM 200 electron microscope.

ABSTRACT

The closed circular forms of DNA of rat liver have been observed during aminoazo-dye carcinogenesis, during regeneration following partial hepatectomy and in control animals. Paucidisperse multiple mitochondrial forms were not observed. Polydisperse smaller molecules were observed following treatment with chemical carcinogens. The cumulative frequency histogram shows the same profile as others observed in HeLa cells. The relative quantity of the small circles to the mitochondrial circles normally present was extremely small. No conclusions could be drawn regarding the source of these molecules. We conclude that neither regeneration nor carcinogenesis results in an alteration of the genetic recombination apparatus of a magnitude which might yield significant quantities of the two aberrant forms of closed circular DNA.

APPROVED: Robert W. Bailey

ROBERT W. BAILEY
LTC, MSC
Commanding

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INTRODUCTION

Circular DNA molecules, because of their circularity, may become inserted into and expelled from a different linear or circular molecule by the standard procedure of genetic recombination. In this insertion stage, their identity as circular molecules is lost. For a recent discussion, see Campbell (1969). Radloff, Bauer and Vinograd (1967) observed that in HeLa cells, a human cancer cell line, aberrant circular DNA forms were present. These forms included paucidisperse multiple length circular mitochondrial DNA molecules as well as polydisperse varying length circular DNA molecules smaller than normal mitochondrial DNA length. One possible explanation of these aberrant forms is an error in the recombination system of the cell. For example, if two circular mitochrondrial DNA molecules recombine, a circular dimer would result. Also, DNAs existing in a prophase-type state might be induced to regain their circular integrities creating the polydisperse class of molecules. Clayton and Vinograd have further observed the appearance of large quantities of aberrant forms of circular DNA - in this case large size molecules - in cells of persons afflicted with a variety of forms of leukemia. In neither case has it been established whether the aberrant molecules preceeded or followed the transformation of these cells into cancer cells. A wide variety of biochemical abnormalities have been observed in cancer cells which, apparently, follow transformation (Reid, 1962). Interest in this particular abnormality centers around the involvement of the genetic material itself and the possibility that the aberrant forms may be intimately associated with the transformation process itself.

We have undertaken a series of experiments in chemical carcinogenesis in an attempt to see whether chemical carcinogens induce aberrant forms of DNA. We have also looked at rapidly dividing cells following partial hepatectomy in the rat to see if the trivial possibility exists that the aberrant forms are the result of cell division.

METHODS AND MATERIALS

The ethidium bromide was a gift of the Boots Pure Drug Co., Ltd., Nottingham, England. Cytochrome C, Equine Heart, was obtained from Calbiochem. Harshaw optical grade cesium chloride was used. All other chemicals

were reagent grade. ^{3}H-thymidine was obtained from New England Nuclear Corporation. A ten-fold diluted solution contained 100 microcuries per ml. A 100 watt Stroblite U. V. spot lamp was used to observe DNA-dye bands.

Male albino caesarian derived Charles River rats weighing 180-200 grams were maintained on Purina chow ad libitum. p-dimethylaminoazobenzene (DAB) was dissolved in acetone and dropped onto the food pellets to produce a final concentration of DAB of 0.06%. The solvent was then allowed to evaporate. This food was used for carcinogenesis experiments. (See Miller and Miller, 1953).

Rats chosen for partial hepatectomy were newly delivered 200 gram rats. For effects of the age of the rat, see Bucher, et al (1964). Rats were two-thirds hepatectomized by excision of the left lateral and median lobes of the liver under pentabarbitol anaesthesia.

Labelling of DNA with tritiated thymidine was accomplished by intraperitoneal injection of isotope at 23 and 25 hours following partial hepatectomy. Sacrifice of the animals occurred two hours after the last injection.

Dialysis tubing is prepared by boiling for 15 minutes in 0.1 M. EDTA, pH 8.0, rinsing the interior of the tubes with distilled water and repeating this cycle. The tubing is finally boiled twice in distilled water with rinsing of the tube interiors. The tubing is stored at 4°C.

EXTRACTION OF RAT LIVER DNA

Extraction of DNA and equilibrium density gradient sedimentation were accomplished using techniques similar to those described by Radloff, Bauer and Vinograd (1967). The medium lobe (or other lobes of comparable mass in the case of partial hepatectomy) of a rat liver was removed under ether anaesthesia. The lobe was minced with scissors and washed in a Buchner funnel with 2.0 M. NaCl, 0.01 M. ethylenediaminetetraacetate (EDTA), pH 8.0.

The chunks of liver were added to 4 ml 0.6% sodium dodecylsulfate (SDS), 0.01 M. EDTA, 0.01 M. Tris, pH 8.0 and mashed in a mortar and pestle for about 15 minutes to obtain a homogeneous mixture. This solution was made up to 12 ml with SDS solution. To this solution was added 2.5 ml 8.0 M. CsCl, and the resultant solution was mixed on a vortex stirrer.

The viscous solution was centrifuged at 17,000 rpm for 45 minutes in 3 polyallomer tubes in an SW50 rotor in a Beckman Model L-2 preparative ultracentrifuge. The liquid layer of about 2 ml between the floating and pelleted

debris was removed from each tube. The combined solution was extracted with cold chloroform.

PREPARATIVE ULTRACENTRIFUGATION

3.65 ml of the DNA solution, 0.1 ml 0.1 M. EDTA, pH 8.0, 0.3 ml 5 mg/ml ethidium bromide were mixed with 3.0 grams CsCl. 3.5 ml of the resultant solution was overlayed with light mineral oil in a cellulose nitrate tube. The DNA was banded at 40,000 rpm for 24 hours in an SW50 rotor.

The major band was located by observation of fluorescence with a U. V. spot lamp. The packed material at the oil interface and most of the major band were removed with a syringe. The tubes were rebalanced with mineral oil and spun an additional 16 hours at 40,000 rpm.

Two distinct fluorescent bands could be observed. The minor, heavier band of closed circular DNA was removed, made up to 1.0 ml with 1.5 M. ammonium acetate and dialyzed into ammonium acetate.

ELECTRON MICROSCOPY

Electron microscopy was performed according to the method of Wetmur, et al. Nine parts of the previously dialyzed DNA solution plus one part 1 mg/ml cytochrome C in distilled water were used as the layering solution. 0.25 M. ammonium acetate was used as the bulk solution. The concentrated stain solution was 0.005 M. uranyl acetate, 0.02 M. HCl in absolute ethanol. The staining solution contained 1 part concentrated stain, 44 parts ethanol, 5 parts water mixed in that order. Samples were mounted on parlodion.

All observations were made with a Phillips EM 200 electron microscope. All photographs were made with an accelerating potential of 60 KV and with a magnification of 5400 onto 35 mm film. Prints were made onto Kodak Kodabromide F-5 paper with an enlargement of 6.8 X.

RESULTS

A series of experiments was undertaken to determine the time following hepatectomy of maximum incorporation of isotope into DNA (see Bucher, et al, 1964). Single doses of isotope were used. Animals were sacrificed two hours later. We found animals (newly delivered) injected at 23 hours to have the greatest label. The study of aberrant circular DNA forms following partial hepatectomy was conducted on such animals. We found no aberrant forms. This

TABLE 1

KNOWN DISTRIBUTION OF ABERRANT CIRCULAR DNA FORMS

Source of Material	Aberrant Circular DNA Forms	
	Small Polydisperse	Large Paucidisperse
1. HeLa Cells (Radloff, Bauer and Vinograd, 1967)	10%	10%
2. Human Leukemia Cells (Clayton and Vinograd 1967)	Not isolated with this technique	more than 10%
3. Rat Liver Cells (this report)		
a. Following 30 days of DAB carcinogenesis	Trace*	None
b. Following 60 days of DAB carcinogenesis	Trace*	None
c. Regenerating cells 25 hours post partial hepatectomy	None	None
d. Control animals	None	None

* The electron micrographs in Figure 1 are those taken after 30 days of DAB carcinogenesis. The same type of distribution was observed after 60 days but not photographed.

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Unclassified

Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)	2a. REPORT SECURITY CLASSIFICATION
US Army Aeromedical Research Laboratory Fort Rucker, Alabama	Unclassified
	2b. GROUP

3. REPORT TITLE

FORMS OF CLOSED CIRCULAR DNA IN RAT LIVER DURING REGENERATION AND FOLLOWING AMINOAZO-DYE CARCINOGENESIS

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (First name, middle initial, last name)

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Charles R. Wilson, SP5

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
June 1969	7	7

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. 3AO 1300 1A 91C	USAARL REPORT NO 69-15
c. Task No. 276	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

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Unclassified

Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
DNA Carcinogenesis						